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Publication date	2017-03-23
Original Citation	Dowling, K. G., Hull, G., Sundvall, J., Lamberg-Allardt, C. and Cashman, K. D. (2017) 'Improved accuracy of an tandem liquid chromatography–mass spectrometry method measuring 24R,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D metabolites in serum using unspiked controls and its application to determining cross-reactivity of a chemiluminescent microparticle immunoassay', Journal of Chromatography A, 1497, pp. 102-109. doi: 10.1016/j.chroma.2017.03.058
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://www.sciencedirect.com/science/article/pii/S0021967317304533 - 10.1016/j.chroma.2017.03.058
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Download date	2023-05-07 22:05:47
Item downloaded from	http://hdl.handle.net/10468/11780

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PII: S0021-9673(17)30453-3
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2017.03.058
Reference: CHROMA 358403

To appear in: *Journal of Chromatography A*

Received date: 30-11-2016
Revised date: 11-2-2017
Accepted date: 19-3-2017

Please cite this article as: Kirsten G.Dowling, George Hull, Jouko Sundvall, Christel Lamberg-Allardt, Kevin D.Cashman, Improved accuracy of an LC-MS/MS method measuring 24R,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D metabolites in serum using unspiked controls and its application to determining cross-reactivity of a chemiluminescent microparticle immunoassay, Journal of Chromatography Ahttp://dx.doi.org/10.1016/j.chroma.2017.03.058

Improved accuracy of an LC-MS/MS method measuring 24R,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D metabolites in serum using unspiked controls and its application to determining cross-reactivity of a chemiluminescent microparticle immunoassay

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HIGHLIGHTS

- An LC-MS/MS method quantifying 4 vitamin D metabolites using LLE was developed
- Quality controls made from pooled, unspiked sera led to improved accuracy
- Method applied to the re-analysis of 159 biobanked sera
- Accurate metabolite measurements were used to explain immunoassay cross-reactivity

ABSTRACT

Measurement of serum 25-hydroxyvitamin D [25(OH)D] is considered the best indicator of vitamin D status. Two minor vitamin D metabolites are common interferences encountered in 25(OH)D assays. The first is 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3], which if not chromatographically resolved from 25-hydroxyvitamin D_3 [25(OH)D_3], can overestimate 25(OH)D concentrations. The second is 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$], which can cross-react with the antibodies in 25(OH)D immunoassays. Our aim was to develop an LC-MS/MS method capable of detecting both 3-epi-25(OH)D_3 and 24R,25$(OH)_2D_3$ in serum without the use of a derivatization agent. We report an isotope dilution LC-MS/MS method, with electrospray ionization in the positive mode, that can simultaneously detect 24R,25$(OH)_2D_3$, 25(OH)D_3, 3-epi-25(OH)D_3, and 25-hydroxyvitamin D_2. The method employs a cost-effective liquid-liquid extraction using only 150 µL of sera and a total run time of 10 minutes. Method performance was assessed by using quality controls made from pooled sera as an alternative to sera spiked with analytes. Biobanked samples, originally analyzed by chemiluminescent microparticle immunoassay (CMIA), were re-analyzed with this method to determine the contribution of 24R,25$(OH)_2D_3$ cross-reactivity to 25(OH)D measurement bias. The CMIA over-estimation of 25(OH)D measurements relative to LC-MS/MS was found to depend on both 25(OH)D and 24R,25$(OH)_2D_3$ concentrations.

KEYWORDS

Vitamin D metabolites; 25-hydroxyvitamin D; LC-MS/MS; Quality control; Immunoassay; Cross-reactivity

1. Introduction

Vitamin D is unique in that it is both produced endogenously through the action of sunlight on the skin, and obtained through the diet. The primary endogenous form of the vitamin produced in the skin and supplied in the diet is vitamin D_3 (cholecalciferol), while vitamin D_2 (ergocalciferol) is a minor dietary-derived form. Each of these vitamers is hydroxylated in the liver to 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2], respectively (**Figure 1**), and are then bound to the vitamin D binding protein and circulate in the blood. The sum of these two metabolites in the circulation is referred to as total 25-hydroxyvitamin D [25(OH)D], which is currently regarded as the best indicator of vitamin D status [1]. Efforts to standardize 25(OH)D measurement across multiple analytical platforms continue through certification and/or accuracy-based quality assurance programs, such as the *Vitamin D Standardization Certification Program* (VDSCP), operated by the Centers for Disease Control and Prevention, and the *Vitamin D External Quality Assessment Scheme* (DEQAS) at Charing Cross Hospital, London [2]. Tandem liquid chromatography-mass spectrometry (LC-MS/MS) has emerged as the preferred analytical technique used for reference measurement procedures [3-6], but immunoassays are widely employed in clinical settings [7].

Two other minor circulating vitamin D metabolites are known to interfere with 25(OH)D assays. An epimer of 25(OH)D_3 [3-epi-25(OH)D_3] (**Figure 1**) is present in the circulation, and if not chromatographically resolved from 25(OH)D_3, may contribute to an over-estimation of total 25(OH)D, particularly in pediatric populations in which the relative concentration of 3-epi-

25(OH)D_3 is greater than those of adults [8, 9]. The first metabolite in the catabolic pathway of 25(OH)D_3 is 24R,25-dihydroxyvitamin D_3 [24R,25(OH)$_2D_3$] (**Figure 1**). As 25(OH)D_3 concentration in serum increases, both24R,25(OH)$_2D_3$ and 3-epi-25(OH)D_3 concentrations increase likewise [7, 8]. The ratio of 24R,25(OH)$_2D_3$ to 25(OH)D_3 has been suggested in recent times as an additional marker of vitamin D status [10-12]. However, 24R,25(OH)$_2D_3$ is also known to interfere with 25(OH)D immunoassays [7]. Through the work of DEQAS, Carter *et al.* [13] have shown that the cross-reactivity of common ligand binding assays to spiked 24R,25(OH)$_2D_3$ varied from <5% to 548%, but that the calculated cross-reactivity from spiked samples did not accurately predict the cross-reactivity of endogenous 24R,25(OH)$_2D_3$ in native samples. This effect of 24R,25(OH)$_2D_3$ on the assessment of total 25(OH)D may explain, at least in part, the recent findings by Cashman *et al.* [14, 15] that have shown a number of immunoassays over-estimate serum 25(OH)D concentrations relative to LC-MS/MS, especially when total 25(OH)D concentrations exceeds 50-80 nmol/L.

There are relatively few LC-MS/MS methods in the literature that quantify both 24R,25(OH)$_2D_3$ and 3-epi-25(OH)D_3. A common strategy is derivatization of the *cis*-diene moiety of vitamin D metabolites with Cookson-type reagents [16-18] to increase sensitivity by improving ionization. Müller *et al.* [19], Satoh *et al.* [20] and Hanson *et al.* [21] have followed this approach using proprietary Ampliflex® [22] or custom-synthesized DAPTAD (4-(4'-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione) and DMEQ-TAD (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione) as the dienophiles, respectively. Non-derivatized methods for vitamin D metabolites that included both 24R,25(OH)$_2D_3$ and 3-epi-25(OH)D_3 have been reported using two-dimensional chromatography by Mena-Bravo *et al.* [23], Baecher *et al.* [24], and Clarke *et al.* [25]. Jenkinson *et al.* [26] have reported an underivatized method using online solid phase extraction (SPE) that analyzes 10 vitamin D metabolites in 8 min, including 24R,25(OH)$_2D_3$ and 3-epi-25(OH)D_3.

Specialist chemical reagents, two-dimensional chromatography, or on-line SPE may not be practical options for many laboratories. In recent times, we developed a LC-MS/MS method capable of measuring 25(OH)D_2, 25(OH)D_3 and its epimer that employed a simple, inexpensive liquid-liquid extraction (LLE), without derivatization, that required minimal training to perform [27]. Our aim in the present work was to incorporate the 24R,25(OH)$_2$$D_3$ analyte into this existing LC-MS/MS method. The type of quality control sample employed proved to be essential for optimal method development. Finally, by accurately quantifying the serum 24R,25(OH)$_2$$D_3$ using this new LC-MS/MS method, we aimed to illustrate how naturally present 24R,25(OH)$_2$$D_3$, via cross-reactivity with antibody in a representative immunoassay, led to inflated estimates of total 25(OH)D.

2. Materials and methods

2.1. Chemical reagents, standards, and columns

All reagents were LC-MS grade unless indicated otherwise. Hexane (HPLC grade), ethanol (HPLC grade), methanol, formic acid, ammonium acetate, and zinc sulfate heptahydrate (reagent grade) were purchased from Sigma-Aldrich (Wicklow, Ireland). Isotopically labelled [^{13}C]$_3$-25(OH)D_2 (50 µg/mL), d_6-25(OH)D_3 (100 µg/mL), d_3-3-epi-25(OH)D_3 (100 µg/mL), d_6-24R,25(OH)D_3 (100 µg/mL), and unlabelled 24R,25(OH)D_3 (100 µg/mL) were purchased from Isosciences as solutions in ethanol (Trevose, PA, USA). The purity of the 24R,25(OH)D_3 standard solution was 97%, as determined by HPLC with PDA detector set to 265 nm. A stock solution of 24R,25(OH)D_3 was prepared from the purchased solution by dilution with ethanol to a concentration of 231.8 nmol/L (adjusted for purity). Certified calibrators SRM 2972a for 25(OH)D_2, 25(OH)D_3 (2 levels), 3-epi-25(OH)D_3 were purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). All of the above standards and stock solutions were stored at -20^0 C. Internal standard

blanks were run daily, and isotope composition remained stable for up to one year. The chromatographic column, Supelco Ascentis® Express F5 100mm x 2.1mm, 2.7µm and guard columns 5mm X 2.1mm, 2.7µm, 3-pk were available from Sigma-Aldrich.

2.2. Preparation of Quality Control material

Standard Reference Materials (SRM) 972a - *Vitamin D Metabolites in Human Frozen Serum* was obtained from NIST. In-house QC sera (Pooled Control I, II and III) were created from pooled sera to represent 3 clinically relevant concentration ranges of 25(OH)D: <30 nmol/L that represents increased risk of vitamin D deficiency [28], >50 nmol/L representing vitamin D adequacy [28], and >75 nmol/L suggested by some to maximize the effect of vitamin D on calcium, bone, and muscle metabolism [29]. Spiked Control A was prepared by spiking Pooled Control I with 25.0 nmol/L of 24R,25(OH)$_2$D$_3$. Bi-level QCs based on lyophilized human serum and enhanced with 3-epi-25(OH)D$_3$/D$_2$ and/or 25(OH)D$_2$ were purchased from Chromsystems (Gräfelfing, Germany); these will be referred to as Spiked Controls B-E.

2.3. Calibration curve

All internal standard (IS) and calibration solutions were prepared daily. Sufficient volume of IS was prepared for addition to the standard curve and to serum samples. The IS spiking solution was prepared from stock solutions to concentrations of 250 ng/mL d_6-25(OH)D$_3$, 125 ng/mL [^{13}C]$_3$-25(OH)D$_2$, and 62.5 ng/mL of d_3-3-epi-25(OH)D$_3$ and d_6-24R,25(OH)D$_3$ in ethanol. A 100 µL aliquot of IS solution was evaporated under nitrogen at room temperature and re-constituted in 1000 µL 32:68 water:methanol for use as the diluent for the preparation of the calibrators. To prepare the highest calibrator, aliquots of NIST 2972a solutions 25(OH)D$_3$ (Level 1), 25(OH)D$_2$, 3-epi-25(OH)D$_3$ and 24R,25(OH)D$_3$ stock solution were evaporated under nitrogen at room temperature and re-constituted in 200 µL IS diluent to give concentrations of 323, 140, 86.6, 73.4 nmol/L of 25(OH)D$_3$, 25(OH)D$_2$, 3-epi-25(OH)D$_3$

and 24R,25$(OH)_2D_3$, respectively. This standard was serially diluted with IS diluent to produce a 7 point calibration curve.

2.4. Sample extraction

To 150 µL serum aliquots, 15 µL of IS solution was added and allowed to stand at room temperature for approximately 15-30 minutes. Vitamin D metabolites were unbound from the vitamin D binding protein and proteins were precipitated with the sequential addition of 150 µL 0.2M aqueous zinc sulfate solution and 300 µL methanol, vortexing after each addition. LLE was performed using 750 µL 30% ethyl acetate in hexane by vortex mixing for 2 minutes and centrifugation for 5 minutes to remove solids. The organic extract was transferred to high recovery HPLC vials and evaporated in a vacuum centrifuge at 30°C, then reconstituted in 100 µL 32:68 water:methanol for injection into the LC-MS/MS.

2.5. LC-MS/MS analysis

Analysis was performed on a Waters Acquity UPLC™ with triple quadrupole mass detector (TQD) using electrospray ionization (ESI) in the positive mode. The column oven temperature was set to 35°C, while the samples were maintained at 10°C. Mobile phase A was 0.1% formic acid, 2 mM ammonium acetate in water, mobile phase B was 0.1% formic acid, 2 mM ammonium acetate in methanol, and the flow rate was 0.45 mL/min. The sample was injected using full loop mode with a 20 µL loop and overfill factor of 3. The initial mobile phase composition was 35% A, 65% B and was maintained for 4.5 min; a non-linear gradient started at 4.5 min and ended at 28% A, 72% B after 8 min; high organic phase 2% A, 98% B flushed the column from 8 to 9.5 min; finally, initial conditions were restored for a total run time of 10 min. The conditions for the Waters Acquity TQD are listed in **Table 1**. A nitrogen generator provided the desolvation gas, while argon was the collision gas.

2.6. Statistics

Statistical analysis was performed with SPSS® Statistics Version 22.0.0.1 (IBM®, USA). Graphical plots were created using SPSS® and Excel® 2013 (15.0.4569.1504; Microsoft®, USA).

3. Method Development

3.1. Improving accuracy of 24R,25(OH)$_2$D$_3$ measurement

Guidelines from the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [30, 31] state that QCs for bio-analytical methods are created by spiking known amounts of analyte into blank biological matrix. In the initial phase of method development and in accordance with these guidelines, blank serum samples, ie. serum samples that contained no detectable 24R,25(OH)$_2$D$_3$ were spiked with 10, 15 or 20 nmol/L 24R,25(OH)$_2$D$_3$. However, sera that naturally contained $\geq$10 nmol/L 24R,25(OH)$_2$D$_3$ also contained a shoulder peak not present in spiked samples.

To illustrate, **Figure 2A** shows the 417.3 > 121.0 MRM chromatogram of a sample measuring 117 nmol/L 25(OH)D$_3$ and 15 nmol/L 24R,25(OH)D$_3$ with a clearly visible shoulder peak. **Figure 2B** is a sample containing 16 nmol/L 25(OH)D$_3$ that was spiked with 15 nmol/L 24R,25(OH)D$_3$ and does not have this interfering peak. The absence of this peak in the low 25(OH)D$_3$ samples spiked with 24R,25(OH)$_2$D$_3$ underestimated the true bias of samples with high 25(OH)D$_3$ and 24R,25(OH)$_2$D$_3$. A modified gradient method (as described in *Section 2.5*) was able to separate this peak, as shown in **Figure 2C.** The mean retention time difference between 24R,25(OH)$_2$D$_3$ and the unknown was 0.60 $\pm$ 0.03 minutes (10 runs over two days).

The area under the curve (AUC) of the unknown peak increased as the 25(OH)D$_3$ increased in the pooled controls (r=0.818, p <0.001). The mean AUC (n=9) of the unknown peak was 28.067, 64.256, and 89.033 for QC I, II, and III, respectively. Comparison of

retention times of known isobaric interferences 23,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D$ [3] eliminated these compounds as the possible identity of the unknown. The biologically active vitamin D metabolite 1,25$(OH)_2D_3$ is hydroxylated at carbon positions 1,3,25 and has a different fragmentation pattern [22] that is not detected in the 417.3 > 121.0 transition. Synthetic vitamin D analogues used to treat vitamin D related pathologies typically have hydroxyl groups at carbons 1 and 3 [32, 33] and likewise would not be observed in this transition.

3.2. Accuracy and selectivity

Pooled sera at three different concentration ranges of 25$(OH)D_3$ (as outlined in *Section 2.2*) were used to assess method performance. Concentration values for 24R,25$(OH)_2D_3$, 25$(OH)D_3$, 25$(OH)D_2$, 3-epi-25$(OH)D_3$ were assigned by measuring each pooled control in duplicate in 3 separate assays. NIST SRM 972a, Level 1-4 was analyzed concurrently with these in-house control sera to verify the accuracy of the measurements. Vitamin D metabolite target values assigned by NIST are provided in **Table 2** [34]**,** and the analyte ranges of these reference sera are similar to those of the pooled controls and individual samples, with the exception of Level 4 which is artificially enhanced with 3-epi-25$(OH)D_3$. The mean bias of each analyte relative to NIST target values was 12.0%, -1.5%, -1.7%, and -4.9% for 24R,25$(OH)_2D_3$, 25$(OH)D_3$, 25$(OH)D_2$, and 3-epi-25$(OH)D_3$, respectively. Specific details of the SRM 972a analysis are discussed elsewhere [35].

Figure 3 shows the overlaid MRM chromatograms of the four vitamin D metabolite standards. Because they have the same mass transitions, 25$(OH)D_3$ and 3-epi-25$(OH)D_3$ peaks are in the same MRM chromatogram, shown in grey. Baseline separation between 25$(OH)D_3$ and 3-epi-25$(OH)D_3$ had been achieved, and while 3-epi-25$(OH)D_3$ and 25$(OH)D_2$ co-eluted, they were different masses and could be analyzed concurrently. No cross-talk

was observed between 3-epi-25(OH)D_3 and 25(OH)D_2 or their isotopically labelled standards.

3.3. Linearity and linear range, sensitivity, stability

The performance characteristics of the LC-MS/MS assay are shown in **Table 3**. The linear ranges correspond to the lower and upper limits of quantitation (LLOQ and ULOQ, respectively). The limits of detection (LOD) were the concentrations at which the signal-to-noise (S/N) ratio of the analyte in serum matrix was no less than 3. The LLOQ was determined by the analyte concentration at which the RSD was 20%. The r^2 values for all calibration curves were $\geq$0.995. The linear range for 24R,25$(OH)_2D_3$ was demonstrated by spiking QC samples with 25 nmol/L (~50% ULOQ) and 55 nmol/L (~75% ULOQ) in duplicate, and spike recovery ranged from 92-109%. Linear ranges of the other 3 analytes had already been established [27]. To test for stability of the analytes, sera were subjected to 3 freeze-thaw cycles or were left at ambient temperature (20-25°C) under amber light for 4 days. No statistically significant differences were seen in the analytes prior to and following the test conditions (data not shown).

3.4. Precision and recovery

Intra-assay precision was determined by 5 replicates of Pooled Controls I-III within a single assay; inter-assay precision was determined by taking duplicate measurements and comparing over three days. The RSD for each analyte is shown in **Table 3**. The mean intra- and inter-assay RSD was less than 15% for all analytes within the limits of quantitation, fulfilling method validation guidelines [30, 31]. Post-extraction recoveries of 8 individual sera are provided in **Table 3.** The lower recovery of 24R,25$(OH)_2D_3$ is due to loss to the aqueous layer on account of its higher polarity, not due to ion suppression of the matrix (which was determined by comparison of IS recovery from extracted serum matrix to IS recovery from extracted phosphate buffered saline blank matrix).

3.5. Measurement of sera from the Finnish Migrant Health and Wellbeing Study

The *Finnish Migrant Health and Wellbeing Study* (referred to as Maamu), a representative sample of 1,310 immigrants that were Russian-speaking or of Somali or Kurdish origin and living in 6 cities in Finland [36], originally assessed serum total 25(OH)D by an Architect Chemiluminescent Microparticle Immuno-Assay (CMIA). As part of a pan-European study, several representative samples, including Maamu, were standardized in terms of their 25(OH)D data [15]. This entailed a subset (*n*=159) of the entire Maamu collection of bio-banked sera being re-analyzed by our original LC-MS/MS method (that did not include 24R,25$(OH)_2D_3$), as described in detail elsewhere [15]. This exercise showed that total 25(OH)D concentrations as measured by CMIA were positively biased compared to that derived by our LC-MS/MS analysis, especially evident at 25(OH)D concentrations above ~80 nmol/L. In the present work, biobanked serum samples with sufficient volume remaining (n=156) were again re-analyzed but with our new LC-MS/MS method that included measurement of 24R,25$(OH)_2D_3$.

4. Results and Discussion

In Sections 2 and 3 above, we report a simple, cost-effective, accurate isotope dilution LC-MS/MS method using LLE that is capable of simultaneously detecting 24R,25$(OH)_2D_3$, 25$(OH)D_3$, 3-epi-25$(OH)D_3$, and 25$(OH)D_2$ in only 150 µL serum without the use of a derivatization agent. We used the re-analyzed Maamu sample subset to test how well pooled controls I-III and spiked controls A-E (see *Section 2.2*) behaved in terms of relationships between 24R,25$(OH)_2D_3$ and 3-epi-25$(OH)D_3$ and their parent metabolite 25$(OH)D_3$ at different concentrations. LC-MS/MS measured serum concentrations of 25$(OH)D_3$, 25$(OH)D_2$, 24R,25$(OH)_2D_3$ and 3-epi-25$(OH)D_3$ in the entire subset of Maamu sample as well as stratified according to low (<30 nmol/L), medium (30-75 nmol/L), and high (>75 nmol/L) 25$(OH)D_3$

concentrations are shown in **Table 4**. Serum 24R,25(OH)$_2$D$_3$ and 3-epi-25(OH)D$_3$, but not 25(OH)D$_2$, increase as the concentration of 25(OH)D$_3$ increases. For four out of the five spiked controls, the relative concentrations of 25(OH)D$_3$, 24R,25(OH)$_2$D$_3$ and 3-epi-25(OH)D$_3$ fell outside the normal proportions observed for unaltered serum. When preparing spiked samples, each metabolite is elevated at concentrations independent of each other. When looking at metabolites within the same pathway, concentrations of each metabolite are not independent but depend on the concentration of the parent compound, in this case 25(OH)D$_3$. By pooling serum at different concentrations of the parent 25(OH)D$_3$, the vitamin D metabolites 24R,25(OH)$_2$D$_3$ and 3-epi-25(OH)D$_3$ maintained their relative proportions to 25(OH)D$_3$ without the need for exogenous spiking.

Thus, by using controls that reflect the relative proportions of both known vitamin D metabolites, but also possibly correlated unknowns, performance assessment of precision and accuracy would more realistically reflect what is observed in real samples. As can be seen in **Table 3**, analyte precision is not constant at different concentrations. If, for example, the precision of 25(OH)D$_2$ measurement was determined using spiked controls B-E with ~40 or ~85 nmol/L 25(OH)D$_2$, as shown in **Table 4**, precision may be estimated to be better than what would be expected from typical samples with a concentration range of 1-16 nmol/L. For the assessment of accuracy, serum spiked with 24R,25(OH)$_2$D$_3$ did not contain an interfering compound present in serum with high 25(OH)D$_3$ concentrations and gave misleading results (see *Section 3.1*). As an alternative to using spiked controls to assess accuracy, we analyzed four levels of NIST SRM 972a, which were also based primarily on unspiked pooled samples (except Level 4, which was spiked with 3-epi-25(OH)D$_3$). Concentration values assigned by NIST for these four vitamin D metabolites were traceable to reference measurement procedures and provided an independent benchmark against which to assess the accuracy of our vitamin D metabolite assay.

With confidence in our new method performance, we endeavored to determine if cross-reactivity to increased levels of 24R,25(OH)$_2$D$_3$ was the primary cause of the positive bias of total 25(OH)D derived by CMIA relative to that from LC-MS/MS measurements within the Maamu sera. The best fit statistical model for predicting total 25(OH)D measured by LC-MS/MS from the total 25(OH)D measured by CMIA by accounting for the concentration of 24R,25(OH)$_2$D$_3$ was:

[Total 25(OH)D by LC-MS/MS] = [Total 25(OH)D by CMIA] + 2.673 * [24R,25(OH)$_2$D$_3$] – 0.034*[Total 25(OH)D by CMIA] * [24R,25(OH)$_2$D$_3$] - 3.859; R^2 = 0.784.

The CMIA -derived total 25(OH)D results were adjusted using this equation, and both adjusted and unadjusted CMIA results relative to LC-MS/MS estimates are plotted in **Figure 4**. The adjusted model predicted total 25(OH)D with closer agreement to LC-MS/MS estimates (R^2 = 0.941) than did the unadjusted CMIA measurements. According to Carter *et al.* [13], the Architect assay for total 25(OH)D had a cross-reactivity factor with 24R,25(OH)$_2$D$_3$ of 548%, calculated from the response to exogenously spiked 24R,25(OH)$_2$D$_3$. Consequently, the calculated over-reactivity was not able to accurately predict the measured CMIA total 25(OH)D. Our model predicted that for DEQAS sample 470 (118 nmol/L total 25(OH)D and 11 nmol/L 24R,25(OH)$_2$D$_3$ as measured by the NIST Reference Measurement Procedure [12]), the Architect CMIA would have a measured value of total 25(OH)D=142 nmol/L. The actual value measured by the CMIA was 146 nmol/L, as reported by Carter *et al.* [13], highlighting how 24R,25(OH)$_2$D$_3$ present in the serum may affect some immunoassay measurements of total 25(OH)D and can lead to dramatically over-inflated estimates of serum total 25(OH)D, particularly in those samples with high serum 25(OH)D$_3$ and thus high 24R,25(OH)$_2$D$_3$ concentrations. The present findings also support earlier suggestions that immunoassays may not react to 24R,25(OH)$_2$D$_3$-spiked samples in the same way as that in native, unspiked samples [10, 13].

We had previously shown that the positive bias of the CMIA-derived estimate for serum total 25(OH)D within Maamu, compared to the LC-MS/MS estimates, became exaggerated at concentrations of 25(OH)D greater than ~90 nmol/L (i.e. the point at which the slope of relation between original CMIA and re-analyzed LC-MS/MS measured values changed; a slope of 1.02 in the original CMIA concentration range of 0-80 nmol/L, and a slope of 0.34 in the concentration range of 90-180 nmol/L [15]). Thus, importantly from a clinical perspective in terms of diagnosis of vitamin D deficiency and insufficiency, below serum total 25(OH)D concentrations of ~90 nmol/L the CMIA and LC-MS/MS analysis yielded relatively comparable estimates; however, above 90 nmol/L there was significant deviation in the estimates. While in this work a relatively small proportion (<5%) of subjects had serum concentrations above 90 nmol/L as measured by CMIA, it is worth noting that the concentration at which this change point occurs can vary by immunoassay, and has been as low as 49 nmol/L [15]. From a public health perspective, the inflation of some immunoassay derived serum total 25(OH)D estimates, as a consequence of cross-reactivity with 24R,25$(OH)_2D_3$, can over-estimate the prevalence of serum 25(OH)D concentrations >125 nmol/L, which are regarded by the IOM committee as being of some reason for concern [28]. Selection of this threshold is based on reported U and reverse J-shaped associations between serum 25(OH)D and adverse consequences, including all-cause mortality, cancer, cardiovascular disease, parathyroid hormone suppression, and intrauterine growth restriction, among others [28,37,38].

5. Conclusion

We have developed a simple, cost-effective LC-MS/MS method using LLE that is capable of simultaneously detecting four vitamin D metabolites in only 150 µL serum. Pooled, unspiked serum used as QC samples more closely reflected the natural proportions of vitamin D metabolites when compared to control serum spiked with analytes, leading to a more accurate

assay. The application of pooled, unspiked QCs could be extended to other metabolic pathways, where relative proportions of each metabolite are under investigation. Quantifying the vitamin D metabolites in relation to each other was important for understanding the nature of cross-reactivity of 25(OH)D immunoassay, which was driven by concentrations of both 25(OH)D and 24R,25$(OH)_2D_3$, not of 24R,25$(OH)_2D_3$ alone. Accurate simultaneous measurement of multiple vitamin D metabolites using LC-MS/MS may not only aid to our understanding of vitamin D biology but also could assist in reducing or correcting for cross-reactivity in immunoassays across the full range of concentrations observed in human serum.

Acknowledgements

Research was supported by funding from the European Union (FP7/2007-2013) under grant agreement no. 613977 (ODIN). Bio-banked sera were provided by the National Institute of Health and Welfare, Helsinki, Finland as part of ODIN.

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Figure 1: Structures and molecular weights of 4 vitamin D metabolites

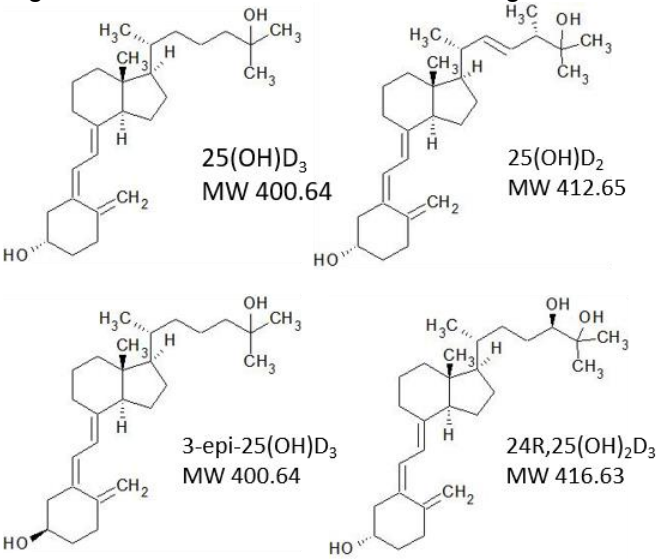

Figure 2: MRM chromatograms of A) native serum with 15 nmol/L 24R,25$(OH)_2D_3$ and unknown interferent peak (arrow); B) serum spiked with 15 nmol/L 24R,25$(OH)_2D_3$; C) new gradient separating interferent (arrow).

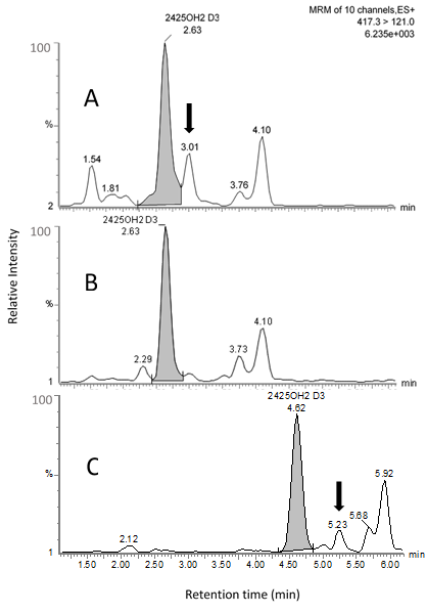

Figure 3: Combined MRM chromatograms with retention times of 24R,25$(OH)_2D_3$, 25$(OH)D_3$, 3-epi-25$(OH)D_3$, and 25$(OH)D_2$ standards. 25$(OH)D_3$ and 3-epi-25$(OH)D_3$ are isobaric and have the same MRM, shown in grey.

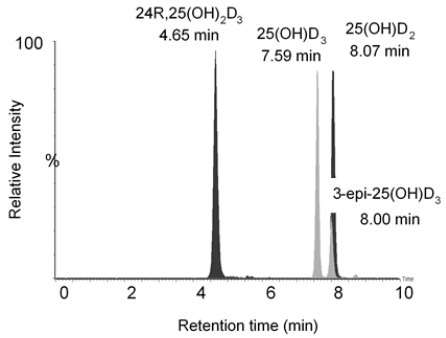

Figure 4: Comparison of sera with total 25(OH)D measured by Architect Chemiluminescent Microparticle Immuno Assay (CMIA) [unadjusted (○) and adjusted for 24R,25(OH)$_2$D$_3$ (▲)] vs. LC-MS/MS. CMIA measurements were adjusted according to the equation [Adjusted CMIA] = [Total 25(OH)D by CMIA]+2.673*[24R,25(OH)2D3]-0.034*[24R,25(OH)$_2$D$_3$]*[Total 25(OH)D by CMIA]–3.859.

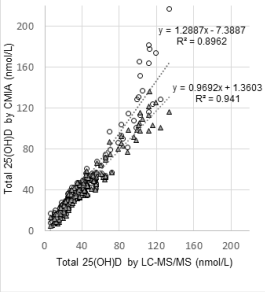

Table 1: Conditions of Triple Quadrupole MS

Source (ES+) parameters		Compound	Transition*	Cone (V)	Collision (eV)
Capillary (kV)	3.2	24R,25(OH)$_2$D$_3$	417.3 → 121.0	21	22
			417.3 → 363.3	21	11
Extractor (V)	2	d_6-24R,25(OH)$_2$D$_3$	423.3 → 121.1	22	20
RF lens (V)	0.5	25(OH)D$_3$**	401.3 → 159.0	24	28
			401.3 → 365.4	22	11
Source Temp (°C)	120	d_6-25(OH)D$_3$	407.4 → 159.1	20	25
Desolvation Temp (°C)	450	d_3-3epi-25(OH)D$_3$	404.4 → 162.0	21	27
Desolvation gas flow (L/hr)	900	25(OH)D$_2$	413.3 → 355.3	23	11
			413.3 → 82.9	23	22
Cone gas flow (L/hr)	30	[^{13}C]$_3$-25(OH)D$_2$	416.2 → 355.2	17	14
Collision gas flow (mL/min)	0.12		All dwell times = 0.2 s		

*quantifier is listed first
**includes 3-epi-25(OH)D$_3$

Table 2: Target concentrations of vitamin D metabolites (nmol/L) in National Institute of Standards and Technology Reference Serum SRM 927a [34]. Concentrations marked with * are certified values, all others are reference values. †Sample spiked with exogenous 3-epi-25(OH)D$_3$

Vitamin D metabolite	Level 1	Level 2	Level 3	Level 4
24R,25(OH)$_2$D$_3$	6.38 ± 0.23	3.39 ± 0.12	3.88 ± 0.13	6.32 ± 0.22
25(OH)D$_3$	*71.7 ± 2.7	*45.1 ± 1.0	*49.5 ± 1.1	*73.4 ± 2.3
3-epi-25(OH)D$_3$	*4.5 ± 0.2	*3.2 ± 0.2	2.9 ± 0.4	*†64.8 ± 5.4
25(OH)D$_2$	1.3 ± 0.2	*2.0 ± 0.2	*32.3 ± 0.8	1.3 ± 0.2

Table 3: Performance characteristics of LC-MS/MS assay

Vitamin D metabolite	LOD (nmol/L)	Linear range	Recovery* (%) mean (SD) n=8	QC Level	Concentration (nmol/L)	Intra-assay RSD (%) n=5	Inter-assay RSD (%) n=3
24R,25(OH)$_2$D$_3$	0.65	1.15-73.4	55 (5)	I	1.7	13.5	3.7
				II	4.1	9.2	8.0
				III	9.0	5.2	9.9
25(OH)D$_3$	0.31	2.52-323	83 (6)	I	28.4	2.6	4.6
				II	55.6	2.2	8.3
				III	87.3	2.8	6.3
3-epi-25(OH)D$_3$	0.17	1.35-86.6	81 (7)	I	<1.35	-	-
				II	1.7	14.3	11.4
				III	3.6	11.0	9.8
25(OH)D$_2$	0.12	2.19-140	86 (8)	I	3.4	10.2	4.0
				II	4.4	6.7	3.0
				III	<2.19	-	-

*Recovery of spiked internal standards from 8 individual sera

Table 4: Serum concentrations of four vitamin D metabolites and selected metabolite ratios from the full subsample (n=156) of *Finnish Migrant Health and Wellbeing Study* (Maamu) participants, and stratified into 3 subgroups based on low, medium and high serum 25(OH)D_3 concentration (<30, 30-75 and >75 nmol/L).

		Vitamin D analyte (nmol/L)					
		25(OH)D_3	**25(OH)D_2**	**24R,25(OH)$_2$ D_3**	**24R,25(OH)$_2$ D_3:25(OH)D_3**	**3-epi-25(OH)D_3**	**3epi25(OH)D_3 :25(OH)D_3**
Native sera* Full set n=156	Mean	42.6	1.7	3.7	0.073	1.5	0.025
	SD	28.7	1.5	4.0	0.029	2.2	0.022
	Range	4.8-134	<0.1-16.4	<0.7-20.5	0.023-0.19	<0.2-12.6	0.002-0.13
Subgroup 1: 25(OH)D_3 <30 nmol/L							
Native sera* n=62	Mean	19.0	1.4	1.1	0.057	0.3	0.02
	SD	6.8	0.7	0.6	0.020	0.3	0.02
	Range	4.8-29.4	<0.1-4.0	<0.7-2.6	0.023-0.12	<0.2-1.3	0.004-0.06
Controls†	Spiked control A*	27.5	3.7	25.4	0.894	1.4	0.049
	Pooled control I**	27.1	3.7	2.2	0.060	1.0	0.048
Subgroup 2: 25(OH)D_3 30-75 nmol/L							
Native sera* n=72	Mean	44.8	2.0	3.4	0.074	1.02	0.022
	SD	10.1	2	1.5	0.020	0.69	0.013
	Range	29.5-70.5	0.6-16.4	1.5-9.5	0.041-0.14	<0.2-4.2	0.002-0.066
Controls†	Spiked control B**	36.8	42.3	3.2	0.087	1.7	0.046
	Spiked control C*	42.2	41.9	3.2	0.076	42.3	1.00
	Pooled control II**	57.6	4.5	4.1	0.071	1.8	0.031
Subgroup 3: 25(OH)D_3 >75 nmol/L							
Native sera* n=22	Mean	102	1.1	12.0	0.12	6.1	0.060
	SD	14	0.7	3.8	0.028	2.7	0.028
	Range	76.6-134	<0.1-3.0	6.7-20.5	0.076-0.19	0.4-12.6	0.005-0.13
Controls†	Spiked control D**	85.2	82.7	3.3	0.039	1.5	0.018
	Spiked control E*	97.2	87.7	2.8	0.029	68.0	0.70
	Pooled control III**	83.6	2.0	9.4	0.11	3.4	0.041

*****Serum measured singly
******Serum measured in duplicate
†For description of control samples, see Section 2.2